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Maternal and Zygotic Expression of the Endoderm-Specific Alkaline Phosphatase Gene in Embryos of the Ascidian, *Halocynthia roretzi*

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Alkaline phosphatase (AP) activity is expressed by endodermal cells of ascidian larvae. It was reported previously that the expression of AP activity is resistant to treatment with actinomycin D, a transcription inhibitor that inhibits the appearance of several other tissue-specific molecules and morphological markers of tissue formation in developing ascidians. The resistance of AP expression to actinomycin D treatment suggests that endodermal AP activity does not depend on zygotic transcription and that its appearance is mediated by the translational activation of maternal AP mRNA present in ascidian eggs. However, it was also shown that anucleate merogons do not develop AP activity. To directly examine whether maternal AP transcripts are present in the cytoplasm of eggs, we isolated a cDNA of an endoderm-specific AP in *Halocynthia roretzi* and examined the temporal and spatial expressions of this gene during embryogenesis using Northern blots and *in situ* hybridization. Maternal AP transcripts were detected in oocytes, cleaving-stage embryos, and in gastrulae, and endoderm-specific AP transcripts dramatically increased about 14 times from the neurula stage to the larval stage in endoderm precursor cells. These results suggest that the differentiation of endoderm is primarily correlated with the activation of zygotic transcription of the AP gene, presumably by egg cytoplasmic factors, similar to how muscle and epidermis are believed to develop. © 1998 Academic Press

Key Words: ascidian embryogenesis; fate determination; endoderm differentiation; alkaline phosphatase gene; cytoplasmic determinants.

INTRODUCTION

Endoderm cells are situated in the central part of the head region of an ascidian tadpole larva. These cells are homogeneous in appearance and contain yolk granules. During embryogenesis, endoderm cells begin differentiation, as indicated by the appearance of an endoderm-specific alkaline phosphatase (AP) (Minganti, 1954). Although the physiological role of AP in endoderm cells is not yet known, the development of AP activity has been used as a tissue-specific marker to investigate underlying mechanisms responsible for endoderm differentiation.

Ascidians are classic organisms for studying how maternal information localized in particular regions of the egg cytoplasm function in the determination of embryonic cell fates (Conklin, 1905). Many studies have demonstrated that most embryonic cell fates are autonomously determined. For example, endodermal AP activity develops in partial embryos derived from isolated presumptive endoderm blastomeres (Whittaker, 1990; Nishida, 1992). Furthermore, dissociated embryonic cells can express AP activity (Nishida, 1992). In cleavage-arrested embryos, AP activity develops exclusively in the cytoplasm of endoderm-lineage cells (Whittaker, 1977; Whittaker and Meedel, 1989). These observations demonstrate the cell autonomy of AP development. When part of a presumptive endoderm blastomere or the vegetal region of an unfertilized or a fertilized egg was fused with an isolated presumptive nonendoderm blastomere, the fused cells sometimes could express AP activity, suggesting that endodermal factors are present in egg cytoplasm and cytoplasm of endoderm progenitor cells (Nishida, 1993; Yamada and Nishida, 1996).

It has been reported using *Ciona intestinalis* and *Styela*

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plicata embryos that treatment with a transcriptional inhibitor, actinomycin D, does not abolish the appearance of AP activity, even when treatment begins prior to fertilization (Whittaker, 1977; Bates and Jeffery, 1987). In contrast, actinomycin D treatment suppresses the expression of other tissue-specific markers of differentiation (Whittaker, 1973; Crowther and Whittaker, 1984). These results suggest that maternal AP mRNA may be already present in the egg cytoplasm and subsequently partitioned into endodermal cells during cleavage, in which case the localized cytoplasmic determinant required for AP development is AP mRNA. However, there is more recent evidence suggesting that the development of AP activity requires zygotic gene transcription. When anucleate egg fragments produced from fertilized eggs were cultured until the controls developed into larvae, the anucleate fragments did not develop AP activity (Bates and Jeffery, 1987; Whittaker and Meedel, 1989; Nishida, 1993). Injection of sperm nuclei into *H. roretzi* anucleate fragments results in AP activity (Bates, 1991). These results suggest that a nucleus is required for AP activity.

To resolve the question of whether ascidian eggs contain maternal mRNA that encodes endodermal AP, we isolated a cDNA of an endoderm-specific alkaline phosphatase, designated *Hr*ES-AP, in *Halocynthia* and investigated the spatial and temporal expressions of this gene using Northern blots and whole mount *in situ* hybridization. Maternal AP transcripts were detected in oocytes, cleaving-stage embryos and in gastrulae, and *Hr*ES-AP mRNA dramatically increased about 14 times from the neurula stage to the larval stage. The results indicate that the appearance of endodermal AP activity primarily depends on the zygotic activation of *Hr*ES-AP genes.

MATERIALS AND METHODS

Ascidians

Adult *Halocynthia roretzi* were purchased during the spawning season from fishermen near Asamushi Marine Biological Station, Tohoku University, Aomori, Japan and from the Ocean Research Institute, University of Tokyo, Iwate, Japan. Naturally spawned eggs were artificially fertilized and reared in Millipore-filtered seawater at 11–13°C. At this temperature, they developed to gastrulae at about 12 h and to the mid-tailbud stage at about 25 h after fertilization. Samples at the appropriate developmental stage were collected by low-speed centrifugation and were fixed for whole mount *in situ* hybridization or quickly frozen in −70°C ethanol for RNA extractions.

Molecular Cloning of HrES-AP

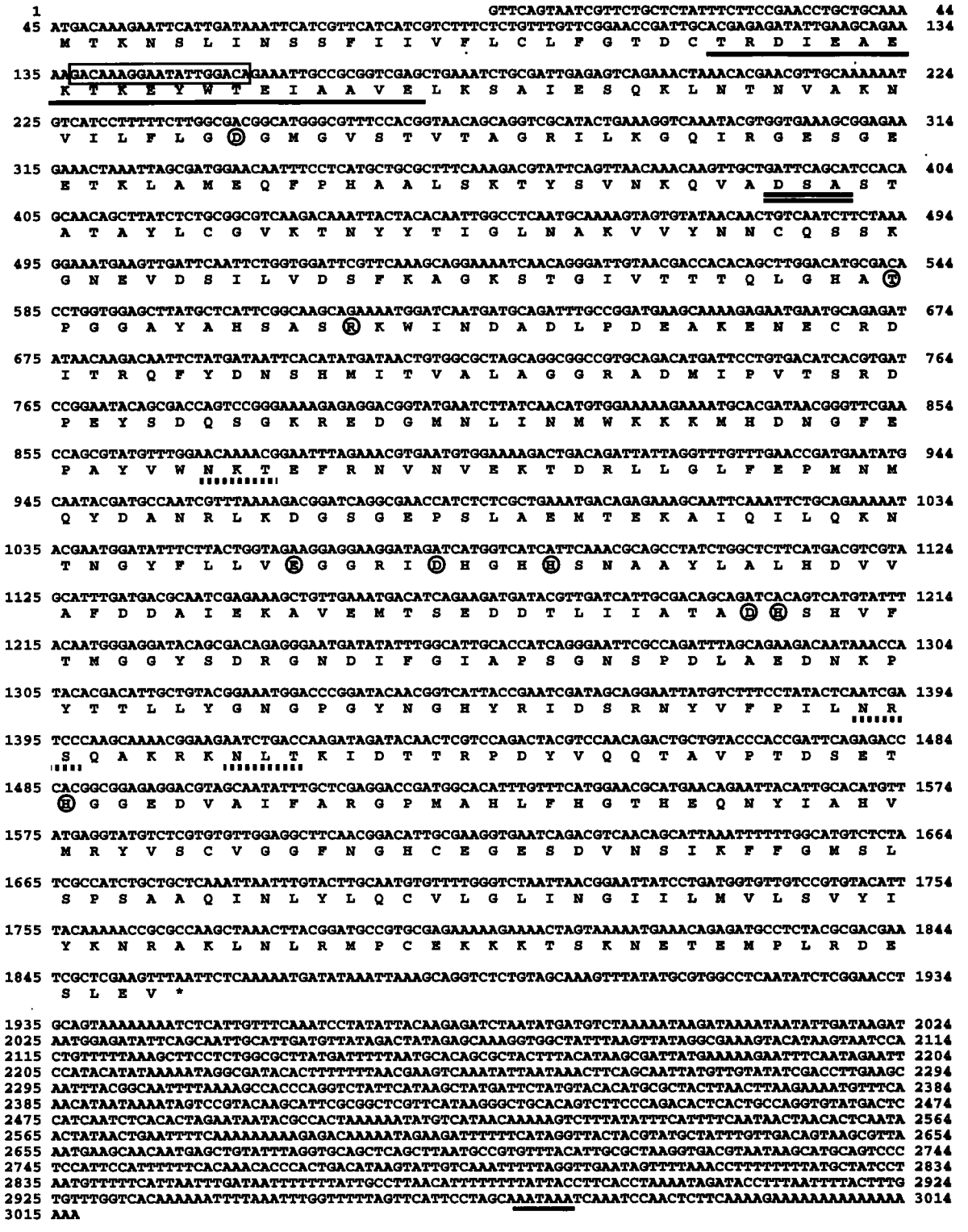
Total RNA was isolated from mid-tailbud embryos using the acid guanidinium thiocyanate–phenol–chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified using Oligotex-dT30 Latex beads (Roche Japan, Tokyo). After reverse transcription to produce single-strand cDNAs, PCR amplification was performed with degenerate primers corresponding to the N-terminal amino acid sequence of *Hr*ES-AP protein (Kumano *et al.*, 1996). The 5′ primer was 5′-GNGA(C/T)AT(A/C/T)GA(A/G)GCNGA(A/G)A-3′, corresponding to the sequence RDIEAEK, and the 3′ primer was 5′-TCNACNGCNGC(A/G/T)AT(C/T)TC-3′, corresponding to the sequence EIAAVE. PCR was carried out for 35 cycles: 95°C, 30 s; 45°C, 1.5 min; 72°C, 20 s. PCR products in the expected size range of 55 bp were then purified using polyacrylamide electrophoresis and subcloned into the pGEM T vector (Promega, Madison, WI) for sequence analysis. The 100% match cDNA of 55 bp with the N-terminal amino acid sequence was identified.

The 19-mer nucleotide (Fig. 1), excluding the primer regions, was used as a probe to screen a cDNA library. The oligonucleotide (5′-GACAAAAGAATATTGGACA-3′) was synthesized by Greiner Japan (Tokyo) and labeled with [γ-^{32}P]ATP (Amersham International, Little Chalfont, England). The cDNA library of the early tailbud embryos that was constructed using the uni-ZAP XR vector (Stratagene, La Jolla, CA) was kindly provided by Dr. N. Satoh (Kyoto University). A total of 1.4×10^5 plaques were screened. Biodyne nylon membranes (Pall, East Hills, NY) were hybridized in a solution containing 6× SSC, 4 mM EDTA, 25 mM KH_2PO_4 (pH 7.2), 5× Denhardt's solution, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA at 37°C for 20 h. Filters were washed twice for 15 min each in 6× SSC at room temperature followed by at 45°C. The hybridized probes were visualized by exposing to an image analyzer (BAS 2000, Fuji). Positive clones were plaque purified twice and an insert from the tertiary screen was *in vivo* excised following Strategene's protocol. The plasmid containing the cDNA of interest was inserted at the *Eco*RI and *Xho*I site of pBluescript (SK$^-$). A clone that was isolated as described above was sequenced using the Auto Read Sequencing Kit (Pharmacia, Uppsala, Sweden) with an ALF DNA Sequencer (Pharmacia). Sequence data from both strands were obtained for the entire *Hr*ES-AP cDNA.

Genomic Southern Analysis

High-molecular-weight genomic DNA was extracted from the adult muscle of *Halocynthia* by the standard procedure (Sambrook *et al.*, 1989). The DNA fragments after digestion with *Sal*I and *Xho*I were electrophoresed on a 0.6% agarose gel, followed by transfer to

FIG. 1. The nucleotide and deduced amino acid sequence of *Hr*ES-AP cDNA clone. The 3017-bp insert includes a single open reading frame that encodes a polypeptide of 604 amino acids. The open box indicates the probe that was used for screening the cDNA library. Both the N-terminal amino acid sequence determined previously (Kumano *et al.*, 1996) and the canonical polyadenylation signal are single underlined. The active site regions predicted from the crystal structure of *E. coli* AP are indicated as follows. The active site, Asp-Ser-Ala, is double underlined. The residue, Arg, coordinated with phosphate and the residues serving as a direct ligand to three metals are circled. The putative N-glycosylation sites are underlined with broken lines. The termination codon is shown by an asterisk. The nucleotide sequence data have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the Accession No. D87872.



a Biodyne nylon membrane by download blotting. The blot was hybridized to ^{32}P-labled (with [α -^{32}P]dCTP (Amersham) using a rediprime DNA labeling system (Amersham)) 1.5-kb *Xho*I fragments (from the 5′ end to a *Xho*I site at nucleotide 1514 of the *Hr*ES-AP cDNA shown in Fig. 1) in the same solution as used in the hybridization for molecular cloning of *Hr*ES-AP for 20 h at 42°C. The membrane was washed twice in 2× SSC and 0.1% SDS at room temperature for 10 min followed by two washes in 0.5× SSC at 55°C for 30 min. The hybridized probes were visualized by exposing to an image analyzer. A 1-kb DNA ladder (Gibco BRL, Gland Island, NY) was used as a molecular mass marker.

Northern Blot Analysis

Total RNA was extracted from various stages of development. Poly(A)$^+$ RNA was purified twice using Oligotex-dT30 Latex beads. Three micrograms of poly(A)$^+$ RNA from embryos at various developmental stages and 10 μg of larval poly(A)$^+$ RNA were electrophoresed on a 1% agarose gel containing 18% formaldehyde. After electrophoresis, RNA was transferred to a Biodyne nylon membrane by download blotting. Samples were hybridized to ^{32}P-labeled 1.5-kb *Xho*I fragments of the *Hr*ES-AP cDNA, as used in Southern blot, in the presence of 50% formamide, 4× SSC, 5× Denhardt's solution, 0.2% SDS, and 0.1% salmon sperm DNA at 42°C for 20 h. The filter was washed twice in 2× SSC and 0.1% SDS at room temperature for 10 min followed by twice in 0.5× SSC at 65°C for 30 min. The hybridized probes were visualized by exposing to X-ray film (Kodak, Rochester, NY) for 2 days. The molecular mass of the *Hr*ES-AP transcript was estimated from the molecular mass markers of a 0.24 to 9.5-kb RNA ladder (Gibco BRL).

Whole Mount in Situ Hybridization

Riboprobes were prepared as follows. A 1.5-kb *Xho*I fragment of *Hr*ES-AP cDNA, as used in Southern and Northern blots, was subcloned in the *Eco*RI–*Xho*I site of the plasmid Bluescript (KS$^+$) for the synthesis of antisense probe and into SK$^-$ for the synthesis of sense probe. The plasmid DNA was digested with *Xba*I for the production of antisense probes or cut with *Xho*I for the synthesis of sense probes and used as a templates for *in vitro* transcription. The RNA probes were synthesized using Boehringer Mannheim DIG RNA labeling kit according to the supplier's instructions. After incubation, the reaction mixture was treated with RQ DNase (Promega) and the transcripts were hydrolyzed by incubation in 42 mM sodium hydrogen carbonate and 63 mM sodium carbonate at 60°C to generate probes with an average length of 500 nucleotides. Whole mount *in situ* hybridization was carried out according to Wada *et al.* (1995). Hybridization reaction was performed at 50°C overnight with hybridization buffer (50% formamide, 5× SSC, 100 μg/ml tRNA, 50 μg/ml heparin, 1% SDS) containing 0.5 μg/ml DIG-labeled antisense or sense probe. RNA hybrids were detected immunohistochemically with alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim). In this procedure, the sense probe gave no significant background after coloring reaction for 2 days at room temperature.

RESULTS

Cloning and Sequence Analysis of the HrES-AP

The 55-bp fragment that matched the N-terminal amino acid sequence of *Hr*ES-AP (Kumano *et al.*, 1996) was obtained by RT–PCR from the mid-tailbud embryos using degenerate primers based on the N-terminal amino acid sequence. The 19-mer nucleotide of the 55-bp fragment was used as a probe to screen cDNA library of early tailbud embryos. A single clone was isolated having a length of 3017 bp (Fig. 1). The nucleotide sequence contains an open reading frame, starting with an initiator methionine at nucleotide 45 and terminating with a stop codon at nucleotide 1859. This open reading frame predicts a polypeptide of 604 amino acids that contains the N-terminal amino acid sequence determined previously (Kumano *et al.*, 1996). The first 23 amino acid residues containing a hydrophobic amino acid cluster likely represents a signal peptide and the three potential N-glycosylation sites were present within the central region of the protein (Fig. 1).

The 13th amino acid of the N-terminal sequence, that could not be determined previously by sequencing amino acid (Kumano *et al.*, 1996), was found to be tryptophan. This amino acid could not be detected using a protein sequencer. The first amino acid of the N-terminal sequence, that was previously considered to be glutamine (Kumano *et al.*, 1996), was found to be threonine in the present study.

A comparison of the amino acid sequence of *Hr*ES-AP with other known protein sequences was analyzed using the BLAST network service of the National Center for Biotechnology Information. *Hr*ES-AP exhibited a 52.6% homology to a nonspecific mouse AP (Terao and Mintz, 1987), a 48.6% homology to a mouse intestinal AP (Manes *et al.*, 1990), and a 48% homology to an embryonic mouse AP (Manes *et al.*, 1990).

Genomic Southern Analysis

We determined the copy number of the genes corresponding to *Hr*ES-AP in the ascidian genome by genomic Southern hybridization. As shown in Fig. 2, only one band was detected in both lanes of *Sal*I (about 7.7 kb) and *Xho*I (about 4.2 kb). This result suggests that *Hr*ES-AP is present as a single copy per haploid genome of *Halocynthia*.

Developmental Expression of HrES-AP: Northern Blot Analysis

*Hr*ES-AP expression during embryogenesis was examined using Northern blot hybridization. A transcript of approximately 3.0 kb, corresponding to cloned *Hr*ES-AP cDNA of 3017 bp, was detected through embryogenesis from oocytes to hatched larvae (Fig. 3). The amount of maternal *Hr*ES-AP mRNA in oocytes, cleaving embryos and gastrulae was relatively low and these transcripts started to accumulate from the neurula to the larval stage. Quantitative measurement of the density of the bands revealed that the amount of AP mRNA increased about 14 times during this period. This result indicates that although maternal AP transcripts are present, the zygotic activation of the *Hr*ES-AP gene contributes to most of the *Hr*ES-AP mRNA pool during embryogenesis.

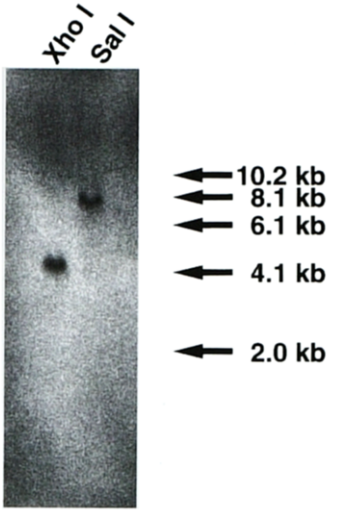


FIG. 2. Genomic Southern blot hybridization. Ten micrograms of digested genomic DNA with *Sal*I and *Xho*I was loaded. The blot was hybridized with ^{32}P-labled 1.5-kb *Xho*I fragments (from the 5′ end to a *Xho*I site at nucleotide 1514 of the *Hr*ES-AP cDNA shown in Fig. 1) and washed twice in 0.5× SSC at 55°C for 30 min. A 1-kb DNA ladder was used as a molecular mass marker.

Spatial and Temporal Expressions of HrES-AP: Analysis by Whole Mount in Situ Hybridization

After extended incubation in the coloring reaction solution, AP signals were not detected in unfertilized eggs and gastrulae (Figs. 4A and 4B). The failure to detect maternal AP transcripts is likely due to its lower concentration in cytoplasm, as suggested by Northern blots. Therefore, spatial localization of maternal AP transcripts is unknown. *Hr*ES-AP transcripts were first detected at the neurula stage. Signals were observed in nuclei of cells in the lateral part of endoderm (Fig. 4C). The number of cells with signal in their nuclei gradually increased in the anterior region of endoderm from the neurula stage to the early tailbud stage (Figs. 4D and 4E). At the mid-tailbud stage, stronger signals were detected in all of the endoderm cells located in the central part of the head region (Fig. 4F). Although maternal mRNA was not detected in the case of *in situ* hybridization (Fig. 4A) in contrast to the result of Northern blot (Fig. 3), these results suggest that the zygotic transcription of *Hr*ES-AP begins at the neurula stage and that the transcripts increased in endoderm precursor cells during the tail elongation. The staining of nuclei shown in Figs. 4C–4E is another indication of initiation of the zygotic transcription.

DISCUSSION

In the present study, a cDNA corresponding to the endoderm-specific AP gene was isolated. *Hr*ES-AP shows 52.6, 48.6, and 48.0% homology to several mouse APs (Terao and Mintz, 1987; Manes *et al.*, 1990). The crystal structure of AP from *Escherichia coli* has been refined and the structures of the active site regions of APs appear to be highly conserved between *E. coli* and mammalian APs (Kim and Wyckoff, 1989; 1991). When the deduced amino acid sequence of *Hr*ES-AP is aligned with that of the *E. coli* AP (24.9% identity) after allowing appropriate deletions and insertions, *Hr*ES-AP seems to have the core of the structure of the *E. coli* enzyme (Fig. 1), including the residue of the active site coordinated with phosphate, and the residues serving as a direct ligand to three metals, two Zn^{2+} and one Mg^{2+}. Therefore, *Hr*ES-AP protein is suggested to retain the AP activity.

A single kind of AP cDNA was obtained from screening an early tailbud cDNA library. However, two types of AP, a major 86-kDa endoderm-specific AP and a minor 103-kDa AP, are previously reported to exist in the membrane fraction of larvae (Kumano *et al.*, 1996). *Hr*ES-AP encodes a major 86-kDa AP because the deduced N-terminal amino acids of *Hr*ES-AP were identical to N-terminal amino acid of 86-kDa AP. Using *Hr*ES-AP probe, only a single band was detected in Northern blot with 10 μg of larval poly(A)$^+$ RNA (Fig. 3) and in Southern blot (Fig. 2). Even under the low stringency conditions, no other band was detected (data

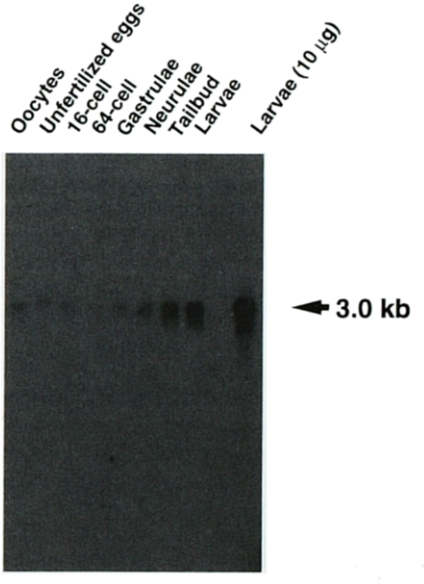


FIG. 3. Expression of *Hr*ES-AP examined by northern blot hybridization. Three micrograms of poly(A)$^+$ RNA from embryos at various developmental stages, as indicated at the top of the blot, and 10 μg of larval poly(A)$^+$ RNA were electrophoresed. The blot was hybridized with ^{32}P-labled 1.5-kb *Xho*I fragments and washed twice in 0.5× SSC at 65°C for 30 min. The length of the *Hr*ES-AP transcript was estimated from the markers of a 0.24 to 9.5-kb RNA ladder.

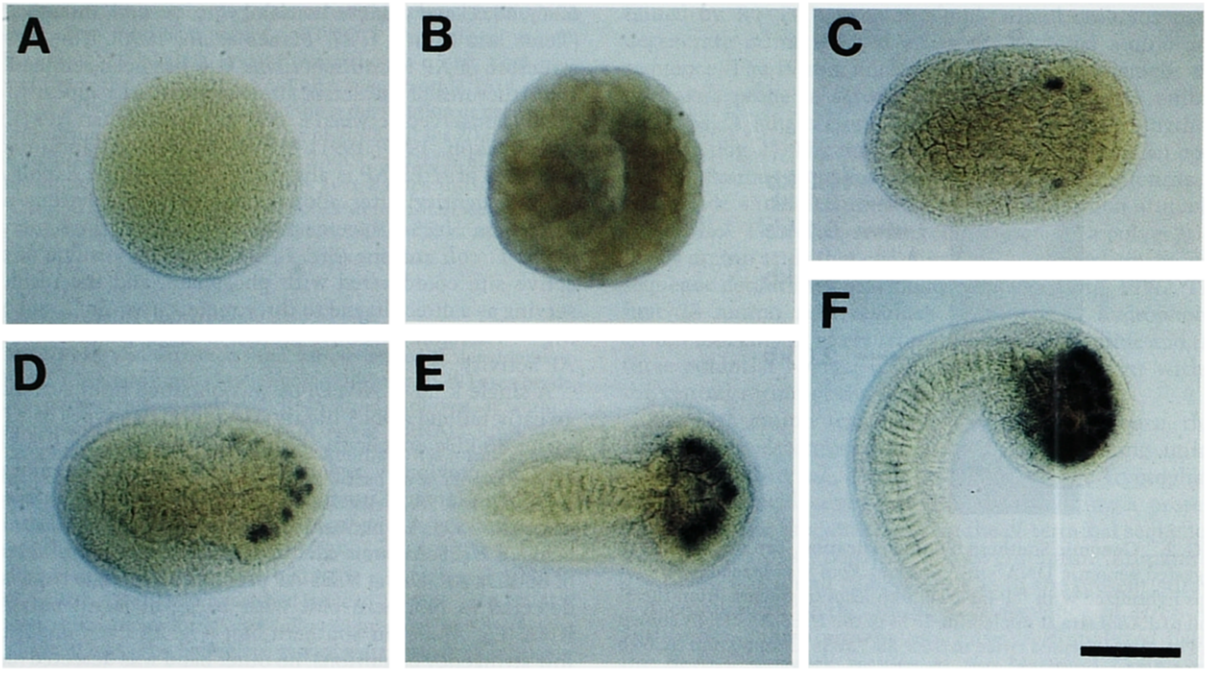


FIG. 4. The temporal and spatial expression patterns of *Hr*ES-AP gene in eggs and embryos was examined using whole mount *in situ* hybridization. (A) An unfertilized egg. (B) An embryo at the gastrula stage, vegetal pole view. (C) An embryo at the neurula stage. Anterior is to the right. (D) More advanced neurula. (E) An embryo at the early tailbud stage. (F) An embryo at the mid-tailbud stage, side view. Scale bar represents 100 μm for all photographs.

not shown). Therefore, it is likely that *Hr*ES-AP probes did not recognize the AP transcripts encoding the 103-kDa enzyme. At the present, we do not know whether the 103-kDa AP is endoderm-specific. It is also not known whether the transcripts for the 103-kDa AP is maternally present. However, the activity of the 103-kDa AP likely contributes less to the total AP activity in a larva because it is present in very small amounts (Kumano *et al.*, 1996). Thus, *Hr*ES-AP activity contributes most, if not all, of the endoderm-specific AP activity in *Halocynthia* larvae.

The present study suggests that small quantities of maternal AP transcripts are present in oocytes and cleaving embryos, and that zygotic transcription of the AP gene is activated at the neurula stage. The transcripts dramatically increased about 14 times from the neurula stage to the larval stage. Therefore, most of the endodermal AP mRNA is likely transcribed from zygotic genes. Zygotic transcription of AP is also supported by the results of *in situ* hybridization experiments. AP signals were detected after the neurula stage. The nuclear staining is another indication of the initiation of transcription. Thus, the differentiation of endoderm appears to be primarily correlated with the activation of zygotic transcription of the AP gene, at least in *Halocynthia* embryos.

In our previous study, the treatment of developing *Halocynthia* embryos with 40 μg/ml of actinomycin D was started at various stages. The sensitive period of appearance of AP activity ended at the gastrula stage. This result suggests that the transcription of *Hr*ES-AP begins at the gastrula stage (Nishida and Kumano, 1997). However, the results of the present study indicate that the transcription of *Hr*ES-AP begins at the neurula stage. One possible explanation is that a certain amount of time is required for actinomycin D to permeate through the embryos. This could cause the sensitive period to end earlier than the start of transcription.

Two differences were observed concerning AP activity in *Halocynthia* embryos compared to *Ciona* and *Styela* embryos. One difference is when AP activities are first detected in endoderm cells during embryogenesis. AP activity first appears at the early tailbud stage in *Halocynthia* as examined using histochemical staining and zymography, and measuring AP activity in homogenized embryos (Nishida and Kumano, 1997). In contrast, it was reported in *Ciona* and *Styela* embryos that AP activity is evident in gastrula stage embryos using histochemical staining (Whittaker, 1977; Bates and Jeffery, 1987). These results suggest that the process of endoderm differentiation begins earlier in *Ciona* and *Styela* embryos than in *Halocynthia* embryos that have eggs almost twice the size as the other species.

The earlier expressions of AP activity in *Ciona* and *Styela* embryos may be correlated with the formation of the endodermal cavity in hatching larvae in these species. In contrast, endodermal cells in *Halocynthia* do not begin gut formation until after hatching.

The second difference pertains to the sensitivity of appearance of AP activity to actinomycin D treatment. The appearance of AP activity could be inhibited in dose-dependent manner when *Halocynthia* eggs were treated with 20, 30, and 40 μg/ml of actinomycin D (Nishida and Kumano, 1997). A small amount of AP activity remained even after treatment with 40 μg/ml. This resistant activity to actinomycin D may be attributed to products from maternal transcripts which was detected in this study.

In contrast, AP activity was not suppressed in *Ciona* by treatment of eggs with 120 μg/ml of actinomycin D (Whittaker, 1977) and in *Styela* by treating eggs with 20 μg/ml of actinomycin D (Bates and Jeffery, 1987). However, AP activities in these experiments were examined only by histochemical staining; that is, they were not measured quantitatively. This leaves the possibility that the resistant activities in these species might be originated from maternal APs, which are present in small amounts in eggs as in the case of *Halocynthia* shown in this study. In a preliminary experiment, we quantified AP activity in *Ciona* embryos treated with actinomycin D. The activity was reduced in a dose-dependent manner, and treatment with 60 μg/ml actinomycin D resulted in one-eighth AP activity of that in normal larvae (Nishida and Kumano, 1997). Moreover, in *Ciona* and *Styela* embryos, there are two reports suggesting the requirement of nucleus for the development of AP activity. When anucleate egg fragments are produced from fertilized eggs, these fragments do not develop AP activity (Bates and Jeffery, 1987; Whittaker and Meedel, 1989). Therefore, the expression of AP activity in endoderm cells appears to depend mainly on zygotic transcription in species other than *Halocynthia*, although small amounts of maternal AP may be present in eggs.

In order to examine directly whether endodermal AP transcripts in *Ciona* embryos are already present in eggs, Northern blot and *in situ* hybridization were also carried out in *Ciona* embryos using *Halocynthia* probes. However, even under conditions of low stringency, no cross-reactivity was detected.

In *Halocynthia*, previous experiments using cell fusion methods suggest that the factors which promote AP expression are present and localized within the egg (Nishida, 1993). These factors are localized in the vegetal pole region after the first stage of ooplasmic segregation and then they are distributed throughout the entire vegetal hemisphere prior to first cleavage. At least in *Halocynthia*, the cytoplasmic determinants responsible for endodermal AP development are not primarily localized endodermal AP transcripts, but unknown factors that promote the transcription of AP genes in endoderm lineage cells.

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